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The conserved carboxyl terminus of human parainfluenza virus type 2 V protein plays an important role in virus growth

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Abstract

Our previous results have shown that some residues of V protein-specific domain in human parainfluenza virus type 2 (hPIV2) are essential not only for STAT protein degradation but also for promoting virus growth. Here, we demonstrated that the virus growth of these recombinant hPIV2s (rPIV2) expressing mutated V proteins were improved in HeLa cell transiently expressing the wild-type V protein, but not in the cells constitutively expressing it. Consequently, we identified the region of the V protein that is essential for its oligomerization and for complex formation with NP protein. We also identified a host protein, AlP1/Alix, involved in apoptosis and efficient budding of several enveloped viruses as an interacting partner of the V and NP proteins. Depletion of AIP1/Alix by small interfering RNA suppressed virus growth. These data suggest that the conserved carboxyl terminus of the V protein plays an important role in virus growth.

Keywords: Human parainfluenza virus type 2; V protein; Virus growth

Introduction

Human parainfluenza virus type 2 (hPIV2) is a member of the *Rubulavirus* genus of the family *Paramyxoviridae*. The *Paramyxoviridae* family includes many well-known human and animal pathogens, such as Sendai virus (SeV), human parainfluenza viruses (hPIV) type 1–4, simian virus 5 (SV5), mumps virus (MuV), Newcastle disease virus (NDV), measles virus (MV), and respiratory syncytial virus, as well as important emerging viruses such as Hendra and Nipah viruses. The negative-stranded RNA genome of hPIV2 is 15,654 nucleotides long and encodes seven viral proteins from six genes (Lamb and Kolakofsky, 2001). The nucleocapsid protein (NP), phosphoprotein (P), and large RNA polymerase (L) protein are important for transcription and replication of the viral RNA genome. The fusion (F) protein and the hemagglutinin-neuraminidase (HN) protein are transmembrane glycoproteins. The matrix (M) protein plays an important role in virus assembly.

The hPIV2 V mRNA is a faithful transcript of the V/P gene, whereas the P mRNA is synthesized through a cotranscriptional RNA editing process in which two nontemplated G residues are inserted into the templated mRNA transcript. Therefore, the N-terminal 164 amino acids (aa) of the V and P proteins are common, while their C-termini are unique (Ohgimoto et al., 1990). The C-terminus of hPIV2 V protein contains seven invariant cysteine residues capable of binding two atoms of zinc and approximately 50% identical among all paramyxovirus V proteins. The hPIV2 V protein is incorporated into virions, as is the case in SV5, MuV, and NDV, while those of SeV and MV are not (Lamb and Kolakofsky, 2001; Mebatsion et al., 2001; Paterson et al., 1995; Takeuchi et al., 1990; Valsamakis et al., 1998).

The hPIV2 V protein appears to be a multifunctional protein. The V protein interacts with the NP and the NP-binding region has been mapped to the N-terminal 47 amino acids (aa), which

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is on the P/V common region (Nishio et al., 1996; Watanabe et al., 1996b). On the other hand, the V protein has two noncontiguous regions, aa 1–46 and aa 175–196, that are required for nuclear localization and retention (Watanabe et al., 1996a). Therefore, it shows diffuse nuclear and cytoplasmic distribution in virus-infected cells. In contrast, as the P protein has two independent NP-binding sites, aa 1–46 and aa 357–395, and a P-multimerization domain, aa 211–248, it is organized in numerous granules with the NP protein in the cytoplasm of virus-infected cells. The difference in the subcellular distribution is due to the binding between residues 357–395 on the C-terminal domain of the P protein and residues 295–402 on the NP protein (Nishio et al., 1996, 1997, 1999b). It is presumed that the P protein forms a complex with both unassembled NP (soluble NP, NP^0) and assembled NP (NP in helical nucleocapsids, NP^{NC}), but the V protein forms a complex only with NP^0 in the case of SeV and SV5 V proteins (Horikami et al., 1996; Randall and Bermingham, 1996). The hPIV2 V protein also binds to viral RNA via its RNA-binding regions, aa 1–82 (P/V common domain) and aa 176–225 (Nishio et al., 2006).

It has recently been demonstrated that viruses of the *Paramyxoviridae*, similar to other viruses, have evolved specific proteins that inhibit interferon (IFN)-induced innate antiviral responses through direct inhibition of cellular STAT proteins. The V proteins encoded by the rubulaviruses, SV5, simian virus 41 (SV41), and MuV, and an avulavirus, NDV, block IFN signaling by targeting STAT1 for degradation (Andrejeva et al., 2002; Didcock et al., 1999a, 1999b; Huang et al., 2003; Kubota et al., 2001; Nishio et al., 2001, 2005b; Park et al., 2003; Ulane and Horvath, 2002; Ulane et al., 2003; Yokosawa et al., 2002; Young et al., 2000), whereas the V protein of hPIV2 targets STAT2 for degradation (Nishio et al., 2001, 2005b; Parisien et al., 2001). The V proteins of MV (a morbillivirus), Nipah, and Hendra viruses (henipaviruses) have been shown to inhibit IFN signaling by preventing STAT1 and STAT2 nuclear accumulation (Palosaari et al., 2003; Rodriguez et al., 2002, 2003; Takeuchi et al., 2003). SeV and hPIV3 (respiroviruses) also block IFN signaling, and this anti-IFN ability has been shown to be a property of their C proteins (Garcin et al., 1999, 2000, 2002; Gotoh et al., 1999, 2003; Kato et al., 2001; Komatsu et al., 2000; Malur et al., 2005; Takeuchi et al., 2001). The rubulavirus V protein-dependent degradation of STAT proteins involves degradation complexes that contain the V protein, STAT1, and STAT2 (and STAT3 in the case of MuV). A number of cellular proteins, including the UV-damaged DNA-binding protein (DDB) 1 and Cullin4A (Cul4A) that are subunits of an SCF-type ubiquitin ligase (Ulane and Horvath, 2002) are also required. The conserved seven cysteine residues of the V protein play a critical role in its specific binding to DDB1 (Andrejeva et al., 2002; Lin et al., 1998). On the other hand, the binding between the V and STAT proteins occurs via tryptophan-rich motif that lies just upstream of the cysteine cluster at the C-terminus, and the cysteine residues are not required for this binding (Nishio et al., 2002). The only paramyxovirus that cannot evade the IFN-induced antiviral responses to data is hPIV4, although it has the V protein, with a highly conserved cycteine-rich domain and tryptophan-rich motif, that binds STAT1, STAT2, DDB1, and Cullin4A (Nishio et al., 2005a). The mechanism of STAT lowering induced by rubulavirus has not clearly been understood yet.

Recombinant morbilliviruses (Schneider et al., 1997), respiroviruses (Delenda et al., 1997; Kato et al., 1997), and an avulavirus (Huang et al., 2003; Park et al., 2003) that cannot express their V and W proteins have been recovered, and all these viruses grow similarly to the respective parent virus at least in some cell lines, such as Vero cells. In contrast to V-minus viruses of these other paramyxovirus genera, we previously demonstrated that the V-minus hPIV2 is highly debilitated and that its growth is very limited even in Vero cells. Recombinant SV5VΔC that lacks the C-terminal V protein-specific domain grows well in Vero cells similarly to parent SV5. However, we also demonstrated that the virus yields of rPIV2VΔC and rPIV2s carrying mutations in the C-terminal V protein-specific domain are two to three orders of magnitude lower than that of wild-type (wt) hPIV2 even in Vero cells (Kawano et al., 2001; Nishio et al., 2005b). The hPIV2 V protein is thus clearly important for promoting virus growth, independent of the anti-IFN activity.

In this study, we investigated whether the virus growth of recombinant hPIV2s (rPIV2) with mutant V proteins would improve in HeLa cells expressing the V protein. Unfortunately, the growth of these rPIV2s did not improve in HeLa cells constitutively expressing the V protein (HeLa-V). However, we demonstrated the improvement of the virus growth in HeLa cells transiently expressing the V protein. As the distribution of viral proteins in the cells infected with these rPIV2s carrying mutations in the V protein-specific domain was different from that in parent hPIV2-infected cells, we searched for the binding partners of V protein and identified that the C-terminus of the V protein was essential for its oligomerization and for complex formation with NP protein. We have also identified a host protein, AlP1/Alix, involved in apoptosis and efficient budding of several enveloped viruses, as an interacting partner of the V protein. These data suggest that the conserved carboxyl terminus of V protein plays an important role in virus growth.

Results

Growth of various rPIV2s with mutant V proteins in the IFN-unresponsive cell lines

In a previous report, we identified hPIV2 V protein residues essential for STAT2 protein degradation in human cell lines that are conserved seven cysteine residues (193,197, 209, 211, 214, 218, and 221 Cys), three tryptophan residues (178, 182, and 192 Trp), a phenylalanine residue (207 Phe) in the C-terminal V-unique domain, and 143 Phe in the P/V common domain. These residues in the V-specific domain that are essential for anti-IFN activity are also essential for promoting virus growth (Nishio et al., 2005b). To confirm the relationship between the virus growth and IFN response, the growth of the recombinant hPIV2s (rPIV2) carrying mutant V protein in the V-specific

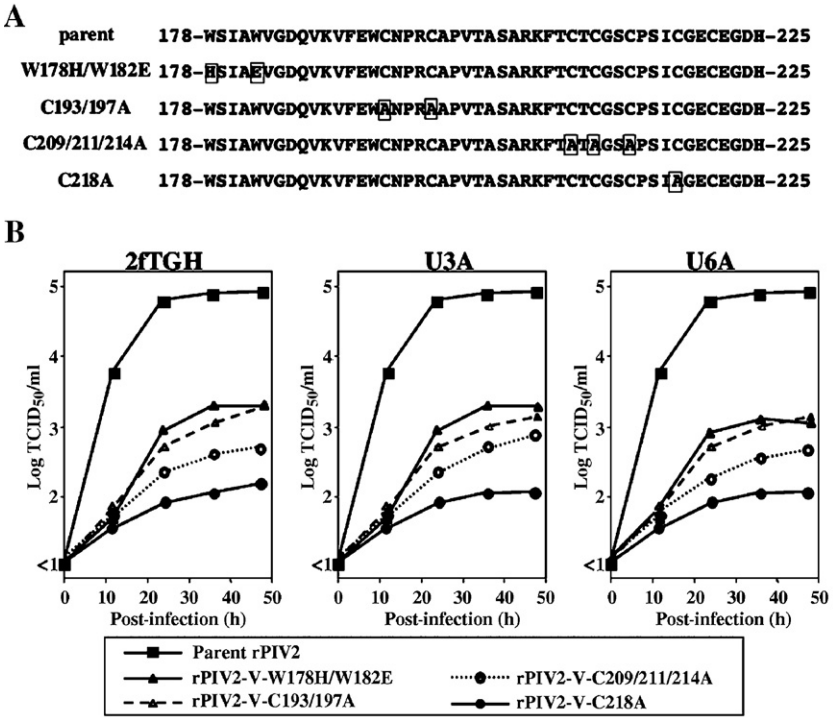

Fig. 1. Growth kinetics of rPIV2s carrying V protein mutations in the IFN-unresponsive cell lines. Monolayers of 2fTGH, U3A (STAT1 deficient), and U6A (STAT2 deficient) cells were infected with the various rPIV2s (A) at an MOI of ca. 0.1. Samples of the supernatants were harvested at various times postinfection, and their titers (Log $TCID_{50}$/ml) were determined by CPE methods on Vero cells (B). The amino acids are numbered from the N-terminus of the V protein.

domain (Fig. 1A) in the IFN-unresponsive cell lines was compared to that in the parental cell line. The U3A and U6A cell lines are defective for STAT1 or STAT2 expression respectively (Leung et al., 1995; McKendry et al., 1991; Muller et al., 1993). The 2fTGH cell line is the parent of the IFN-unresponsive daughter cell lines (Pellegrini et al., 1989). Virus growth rates of parent rPIV2 in 2fTGH, U3A, and U6A cells are similar. In contrast, the growth rates of mutant rPIV2s are about two to three orders of magnitude lower than those of parent rPIV2 even in U3A or U6A cells (Fig. 1B), showing that both the Typ motif and the Cys cluster are important for the V protein to promote virus growth in an IFN-independent manner.

Growth of various rPIV2s with mutant V proteins in the HeLa cell lines expressing the V protein

Subsequently, we performed a complementation assay to examine whether the constitutive expression of the V protein would enhance the growth of the mutant rPIV2s. The HeLa-V cell line is derived from the HeLa cells and constitutively expressing the hPIV2 V protein (Nishio et al., 2001). Virus yields of parent rPIV2 in HeLa and HeLa-V cells at 48 h postinfection were similar. However, the yields of mutant rPIV2s were about three orders of magnitude lower than that of parent rPIV2 even in these HeLa-V cells (Fig. 2A). These results demonstrated that the constitutive expression of the V protein could not improve the growth of the mutant rPIV2s.

Since the constitutively expressed V protein has no effect on the virus growth, we next generated a stable HeLa tet-on cell line harboring a doxcycline (Dox)-inducible hPIV2 V cDNA (HeLa-tet/V) as described in Materials and methods. As shown in Fig. 2B, the V protein was detected by Western blotting after appropriate stimulation with 5 μg/ml Dox. The amount of the V protein in the HeLa-tet/V cells cultured for 2 h in the presence of Dox is similar to that in the HeLa cells at 15 h postinfection. The virus yields of mutant rPIV2s in HeLa-tet/V cells treated with Dox simultaneously or at 14 h postinfection improved 10 to 100-fold than those in the nontreated cells (Fig. 2C). The virus yields in the cells treated with Dox at 14 h postinfection were significantly higher than those in the cells simultaneously treated with Dox (Fig. 2C). These results indicate that the transiently expressed V protein has an ability to promote virus growth.

To investigate whether other V proteins of rubulaviruses have the same ability, we established the HeLa tet-on cell lines harboring Dox-inducible HA-tagged V cDNAs of hPIV2, SV41, SV5, and MuV (Fig. 3A). As described above, the virus yields were measured in these tet-on HA-tagged V protein-expressing cell lines. As shown in Figs. 3B–E, the SV41 V protein exhibited an ability to promote hPIV2 growth like the hPIV2 V protein, while SV5 and MuV V proteins did not.

Mapping of the domains on the V and NP proteins required for V–NP interaction

In previous studies, we identified a domain on the V protein of hPIV2 responsible for binding to the NP; that is, amino acid residues 1–46 of the P/V common region are required for

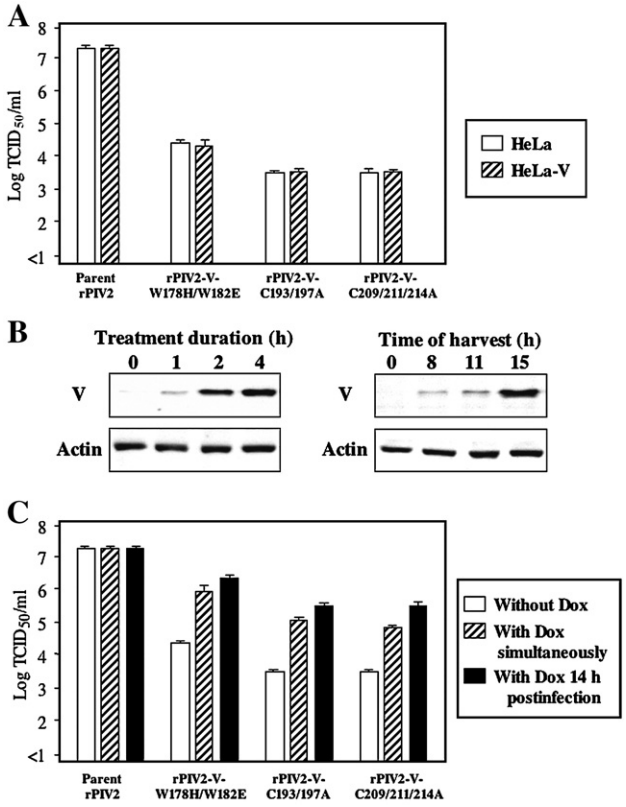

Fig. 2. Virus growth of rPIV2 carrying V protein mutations in the HeLa cell lines, in which hPIV2 V protein is expressed constitutively or induced by Dox-inducible manner. (A) Virus growth of parent rPIV2, rPIV2-V-W178H/W182E, -C193/197A, or -C209/211/214A in HeLa cells or HeLa-V cells, which are a cell line constitutively expressing hPIV2 V protein. Forty-eight hours postinfection, supernatant was harvested and used for virus titration. Virus titers were determined as described in the legend for Fig. 1B. (B) HeLa-tet/V cells, which are a cell line harboring Dox-inducible hPIV2 V cDNA, were treated for various times with 5 μg/ml Dox (left panel). HeLa cells infected parent rPIV2 at an MOI of 2 were harvested at various times (right panel). Total protein extracts were probed by Western blots using anti-P/V mAb. Actin was used as a loading control. (C) Virus growth of parent rPIV2, rPIV2-V-W178H/W182E, -C193/197A, or -C209/211/214A in HeLa-tet/V cells treated with/without 5 μg/ml Dox at 48 h postinfection. Virus titers were determined as described in the legend for Fig. 1B.

binding to the aa 401–493 region on the NP (Nishio et al., 1996, 1999b; Watanabe et al., 1996b). The NP and P protein in rPIV2 wt-infected cells showed fine and numerous granules, but these proteins in the cells infected with rPIV2s that have mutations in the V-specific region distributed diffusely and some masses of these proteins were found (data not shown). It has been suggested that the V protein keeps NP soluble prior to encapsidation in the SV5 system (Precious et al., 1995). Therefore, it is presumed that the C-terminal region of V protein has another binding domain to other virus protein(s) and a role in keeping NP soluble. To identify the putative binding domain in addition to that in the N-terminal region of the V protein, N-terminally truncated V protein, V83-225, and NP were expressed in bacterial cells and purified as described in Materials and methods. The mixture of V83-225 protein and NP was immunoprecipitated with anti-NP monoclonal antibody (mAb) 20A or anti-P/V mAb 85A (this mAb react with aa 115–154 on the P/V common domain) and then the immunoprecipitates were analyzed by Western blot assay. As shown in Fig. 4B (right panel, line 1), the complex of NP and V83-225 was detected in the immunoprecipitates with either anti-NP or anti-P/V antibody. Since both the N- and C-terminally truncated V protein, V83-175, could not make the complex (Fig. 4B, lane 2), the C-terminal 50 amino acids of V protein seemed essential for the V–NP complex formation. To identify the amino acid essential for the complex formation, four mutants were constructed in the V83-225 background as illustrated in Fig. 4A, 3–6. The complex of NP and V83-225/W178E/W182E (Trp-motif mutant) was not detected by anti-NP mAb or anti-P/V mAb (Fig. 4B, lane 3). However, the internally deleted mutant V83-225/Δ155–192 that does not have the Trp-motif could make a complex with NP protein, although the amount of complex immunoprecipitated with anti-P/V mAb was fewer (Fig. 4B, lane 6). The mutants of Cys-motif, V83-225/C193/197A and V83-225/C209/211/214A, could make the complex, but these immunoprecipitates with anti-P/V mAb were also

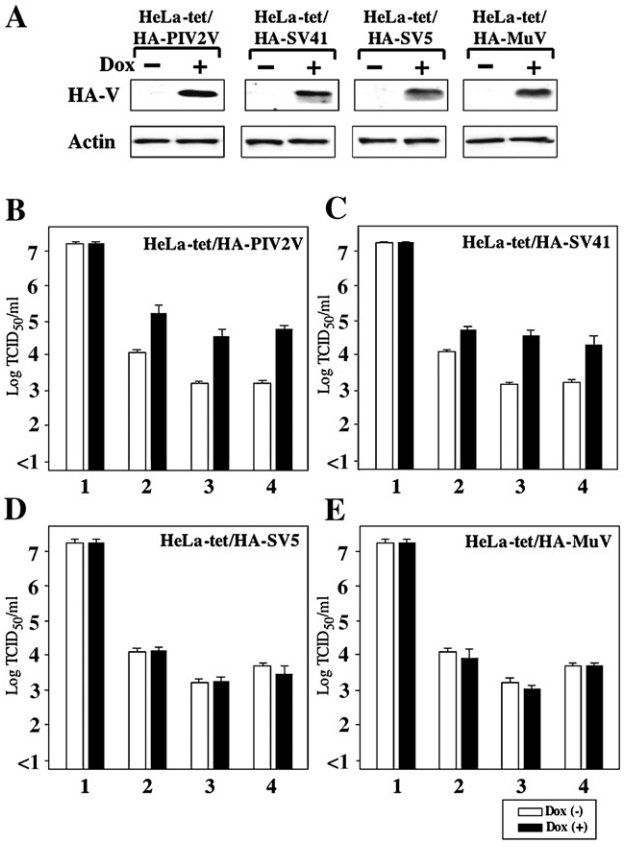

Fig. 3. Virus growth of rPIV2s in HeLa-tet cell lines, in which various HA-tagged V proteins are induced by treatment with Dox. (A) HeLa-tet cell lines harboring Dox-inducible various HA-tagged V cDNA were treated for 24 h with/without 5 μg/ml Dox. Total protein extracts were probed by Western blots using anti-HA mAb. Actin was used as a loading control. (B–E) Virus growth of rPIV2s in HeLa-tet/HA-PIV2V (B), HeLa-tet/HA-SV41 (C), HeLa-tet/HA-SV5 (D), and HeLa-tet/HA-MuV (E) cells treated with/without 5 μg/ml Dox at 48 h postinfection. Lane 1; parent rPIV2, lane 2; rPIV2-V-W178H/W182E, lane 3; rPIV2-V-C193/197A, and lane 4; rPIV2-V-C209/211/214A at an MOI of ca. 0.1. Virus titers were determined as described in the legend for Fig. 1B.

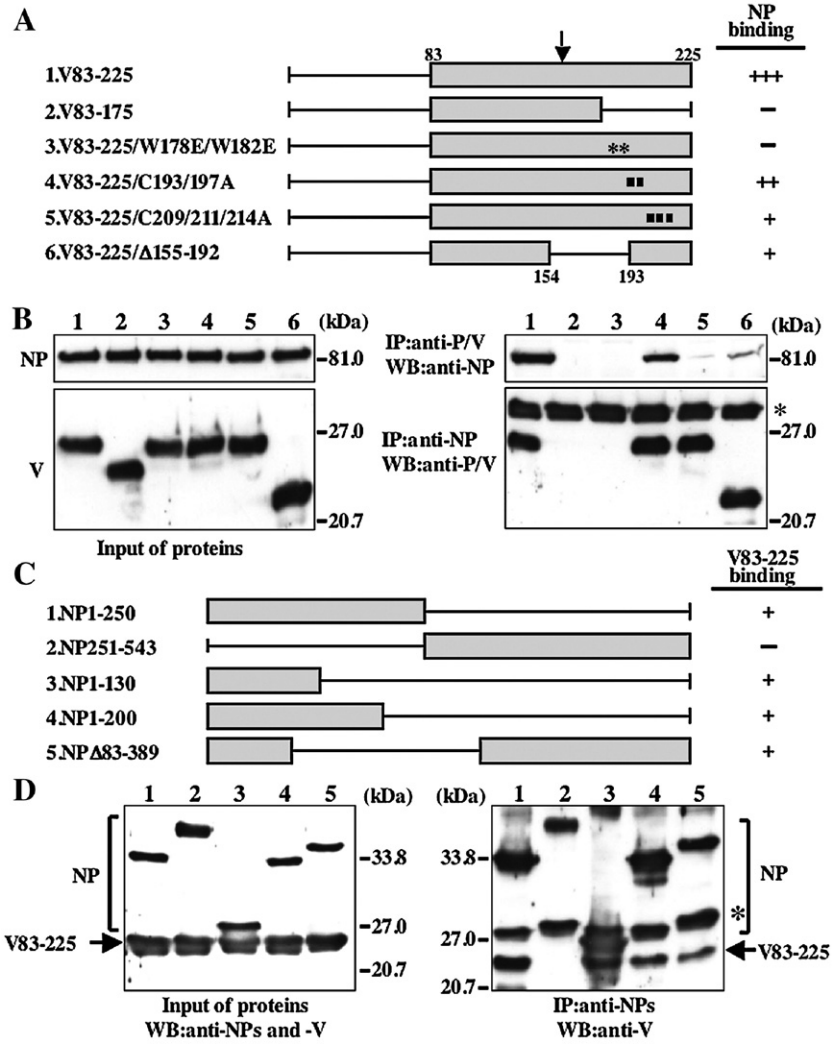

Fig. 4. Analysis of interactions between V and NP proteins by immunoprecipitation and Western blot assay. (A) Schematic diagram of the V proteins. The asterisks indicate the position of the mutation residues of Trp-motif. The closed squares indicate the position of the mutation residues of Cys-motif. The arrow above marks the editing site. Binding with NP protein is summarized on the right panel. (B) Western blot analysis of the purified proteins from transfected *E. coli* cells (input of proteins, left panel) and of the proteins from immunoprecipitates using anti-P/V (upper, right panel) or anti-NP (lower, right panel) mAb. Numbers on the top of the figure correspond to the V proteins as described in A. The asterisk on the right indicates the immunoglobulin light chain. (C) Schematic diagram of the NP proteins. Binding with V83-225 protein is summarized on the right panel. (D) Western blot analysis of the purified proteins from transfected *E. coli* cells (input of proteins, left panel) and of the proteins from immunoprecipitates using anti-NP mAbs (right panel, lanes 1, 3, and 4: anti-NP mAb 366-1, lanes 2 and 5: anti-NP mAb 159-1). Numbers on the top of the figure correspond to the NP proteins as described in C. The asterisk on the right indicates the immunoglobulin light chain.

fewer (Fig. 4B, lanes 4 and 5). The results from these co-immunoprecipitation studies summarized in Fig. 4A indicate that the C-terminal 50 amino acids on the V protein is essential for its binding with NP protein and that conserved Cys-motif and Trp-motif are also important. Especially, it is speculated that the mutation of Trp-motif on the V protein may alter the conformation of the V protein.

Next, we intended to determine the domain on the NP that would be responsible for its binding to the C-terminus of the V protein by using the similar method as described above. Various truncated NPs as illustrated in Fig. 4C were mixed with V83-225 and then the mixtures were immunoprecipitated with anti-NP mAbs (Fig. 4D). As shown Figs. 4C and D, V83-225 and the truncated NPs containing the N-terminal 82 amino acids were co-immunoprecipitated.

Thus, as summarized in Figs. 4A and C, the C-terminal 50 amino acids on the V protein are important for its binding to the N-terminal 82 amino acids on the NP.

Oligomerization of the V protein

To examine the possible interaction between the V and P proteins, the Flag-tagged V protein and non-tagged P protein were coexpressed in COS cells and the cell lysates were immunoprecipitated by anti-Flag mAb as described in Materials and methods. As shown in Fig. 5B, lane 6, the non-tagged P and tagged V proteins were not coprecipitated, indicating that the P protein could not bind directly to the V protein. Thus, to test the possible homologous interaction between the V proteins, we did the same assay. As shown in

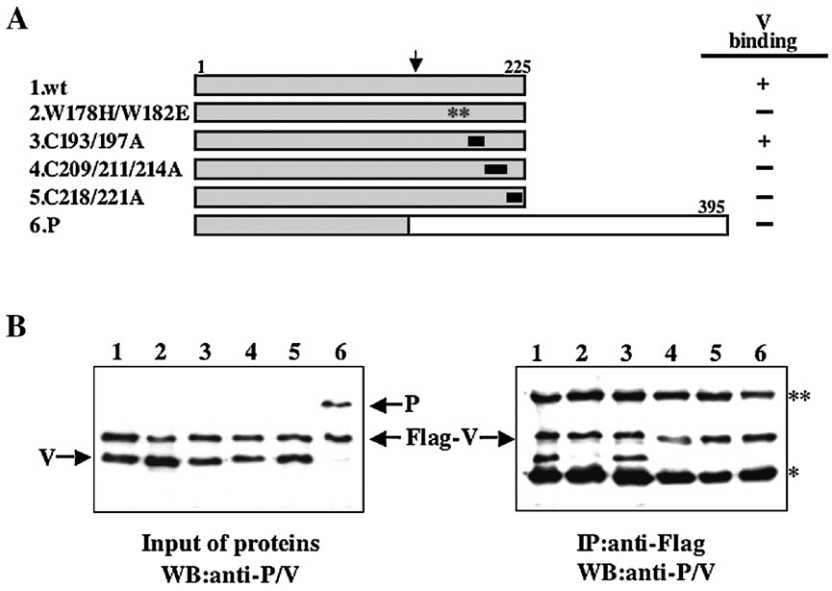

Fig. 5. Oligomerization of the V protein. (A) Schematic diagram of the V and P proteins. The asterisks indicate the position of the mutation residues of Trp-motif. The closed squares indicate the position of the mutation residues of Cys-motif. The arrow above marks the editing site. The shaded box on the P protein indicates the P/V common domains. The P-specific domain arises due to the insertion of two nontemplated-encoded G residues and is indicated by the open box. Binding with V protein is summarized on the right panel. (B) Western blot analysis of the proteins from transfected COS cells lysates (input of proteins, left panel) and from immunoprecipitates using anti-Flag mAb (right panel). Numbers on the top of the figure correspond to the V and P proteins as described in A. One or two asterisks on the right indicate the immunoglobulin light or heavy chain, respectively.

Fig. 5B, lane 1, the non-tagged V and tagged V protein were coprecipitated, indicating that the V protein-specific region is critical for self-association. To identify the amino acid(s) essential for the self-association, various mutant V proteins were constructed as illustrated in Fig. 5A. Although all the polypeptides were successfully expressed in the transfected cells (Fig. 5B, left panel), only C193/197A V protein was coprecipitated by anti-Flag mAb (Fig. 5B, right panel, lane 3) and all other mutant V proteins were not coprecipitated (Fig. 5B, right panel, lanes 2, 4, and 5). These data indicate that the C-terminal 28 aa on the V-specific region are important for self-association and that mutation of Trp-motif loses the function for self-association, suggesting that it alters the conformation of V protein.

The V and NP proteins of hPIV2 bind with AIP1/Alix

SeV C protein has anti-IFN capacity and binds to a host protein, AIP1/Alix, that is involved in apoptosis (Sakaguchi et al., 2005). hPIV2 and SV5 V proteins also have anti-IFN activity and SV5 V protein is important for blocking apoptosis (Sun et al., 2004). Therefore, we presumed that AIP1 would bind to hPIV2 V protein. GST and N-terminally GST-tagged AIP1 proteins were expressed in *E. coli*, purified, and subjected to GST-pulldown assay. The hPIV2-infected cell lysate was allowed to bind to the glutathione–Sepharose 4B beads alone (lanes B), Sepharose-immobilized GST (lanes C), or Sepharose-immobilized GST-AIP1 (lanes D) for 8 h followed by an analysis of the bound viral proteins by Western blotting. As shown in Fig. 6A, lanes 4 and 8, the NP and V proteins were detected in association with GST-AIP1, while the P protein was not. Furthermore, binding between GST-AIP1 and M proteins was not detected by this method (data not shown). To make sure that no other viral proteins are needed for these associations, the NP-, P-, or V-expressed cell lysates were investigated by using similar method. The NP and V proteins were detected in association with GST-AIP1 without other viral proteins (Fig. 6B, lanes 4 and 12), while the P protein was not with/without other viral proteins (Figs. 6A, lane 8 and B, lane 8).

To identify the domain on the V protein responsible for its binding to AIP1, various V proteins as illustrated in Fig. 6C were tested. The V proteins that have mutations in Cys-motif and Trp-motif could not bind to GST-AIP1 (Fig. 6D). These data suggest that the V-specific region is important to bind with AIP1.

The hPIV2 V alters its distribution when coexpressed transiently with AIP1

The wt V protein was detected exclusively in the nuclei (Fig. 7A), while the C-terminally HA-tagged AIP1 protein was detected exclusively in the cytoplasm (Fig. 7B) when they were expressed transiently. Since the V protein can bind to AIP1 as described above, we performed cotransfection experiment in order to see whether the AIP1 would affect the subcellular distribution of the V protein. The AIP1-HA protein in the cells expressing only AIP1-HA was found in the cytoplasm (the left cell, Fig. 7C), and the wt V protein in the cells expressing only V protein was found in the nuclei (two cells in the right corner of Fig. 7C). However, the wt V protein in the cells expressing both the wt V and AIP1-HA

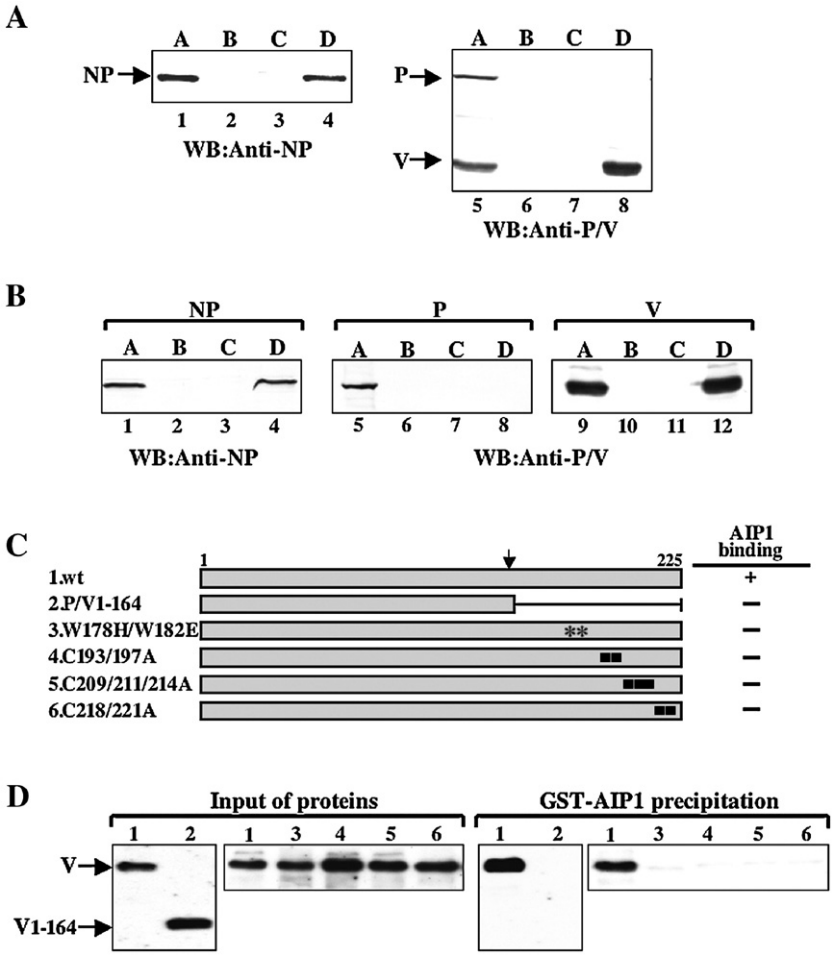

Fig. 6. Analysis of interactions of NP and V proteins with AIP1 by GST-pulldown and Western blot assays. (A) Western blot analysis of the proteins by using anti-NP (lanes 1–4) or anti-P/V (lanes 5–8) mAb. Lanes A are input of proteins from parent rPIV2-infected Vero (lanes 1 and 5). Lanes B, C, or D are precipitates from GST-pulldown assay of glutathione–Sepharose 4B beads alone, glutathione *S*-transferase protein, or GST-AIP1 fusion protein, respectively. (B) Western blot analysis of the proteins by using anti-NP (lanes 1–4) or anti-P/V (lanes 5–12) mAb. Lanes A are input of proteins from transfected COS (lanes 1, 5, and 9) cells lysates. Lanes B, C, or D are precipitates from GST-pulldown assay of glutathione–Sepharose 4B beads alone, glutathione *S*-transferase protein, or GST-AIP1 fusion protein, respectively. Lanes 1–4, 5–8, and 9–12 are transfected with an expression plasmid for hPIV2 NP, P, or V, respectively. (C) Schematic diagram of the V proteins. The asterisks indicate the position of the mutation residues of Trp-motif. The closed squares indicate the position of the mutation residues of Cys-motif. The arrow above marks the editing site. Binding with AIP1 protein is summarized on the right panel. (D) Western blot analysis of the proteins from transfected COS cells lysates (input of proteins, left panel) and from GST-pulldown precipitates (right panel). Numbers on the top of the figure correspond to the V proteins as described in C.

proteins (arrow pointed) was found in the cytoplasm (Fig. 7C). On the other hand, the mutant V protein, V-C209/211/214A, in the cells expressing only mutant V protein was found in the nuclei like the wt V protein (the cell in the left corner of Fig. 7D), but the mutant V protein in the cells expressing both the V and AIP1-HA proteins (arrow pointed) was kept exclusively in the nuclei (Fig. 7D). Other mutant V proteins, V-C193/197A, V-C218/221A and V-W178H/W182E, were also found in the nuclei, even if both the mutant V and AIP1-HA proteins were expressed in the same cells (data not shown). When the N-terminally HA-tagged AIP1 was expressed together with the hPIV2 V protein, we got the same result to that using AIP1-HA (C-terminally HA-tagged AIP1) (data not shown).

We then established new cell lines constitutively expressing both AIP1-HA and hPIV2 V proteins (HeLa/AIP-HA+V). As shown in Fig. 7E, localization of the AIP1-HA protein in HeLa/AIP-HA+V cells was detected in the cytoplasm but the stably expressed V protein was detected in the nuclei. Thus, these data suggested that AIP1 alters the subcellular distribution of the transiently expressed V protein, but does not that of the stably expressed V protein. Thus, nuclear localization of the transiently expressed mutant V proteins seems consistent with their inability to bind with AIP1.

Suppression of parent virus growth by AIP1 depletion

To investigate possible involvement of AIP1 in hPIV2 virus growth, we established new cell lines, HeLa-tet/shAIP1-1 or 1-2, in which were depleted the intracellular AIP1 by using short hairpin RNA (shRNA) induced by adding Dox as described in Materials and methods. As shown in Fig. 8A, AIP1-targeted shRNAs effectively suppressed the expression of HA-tagged AIP1, indicating that the intrinsic AIP1 was also depleted. By

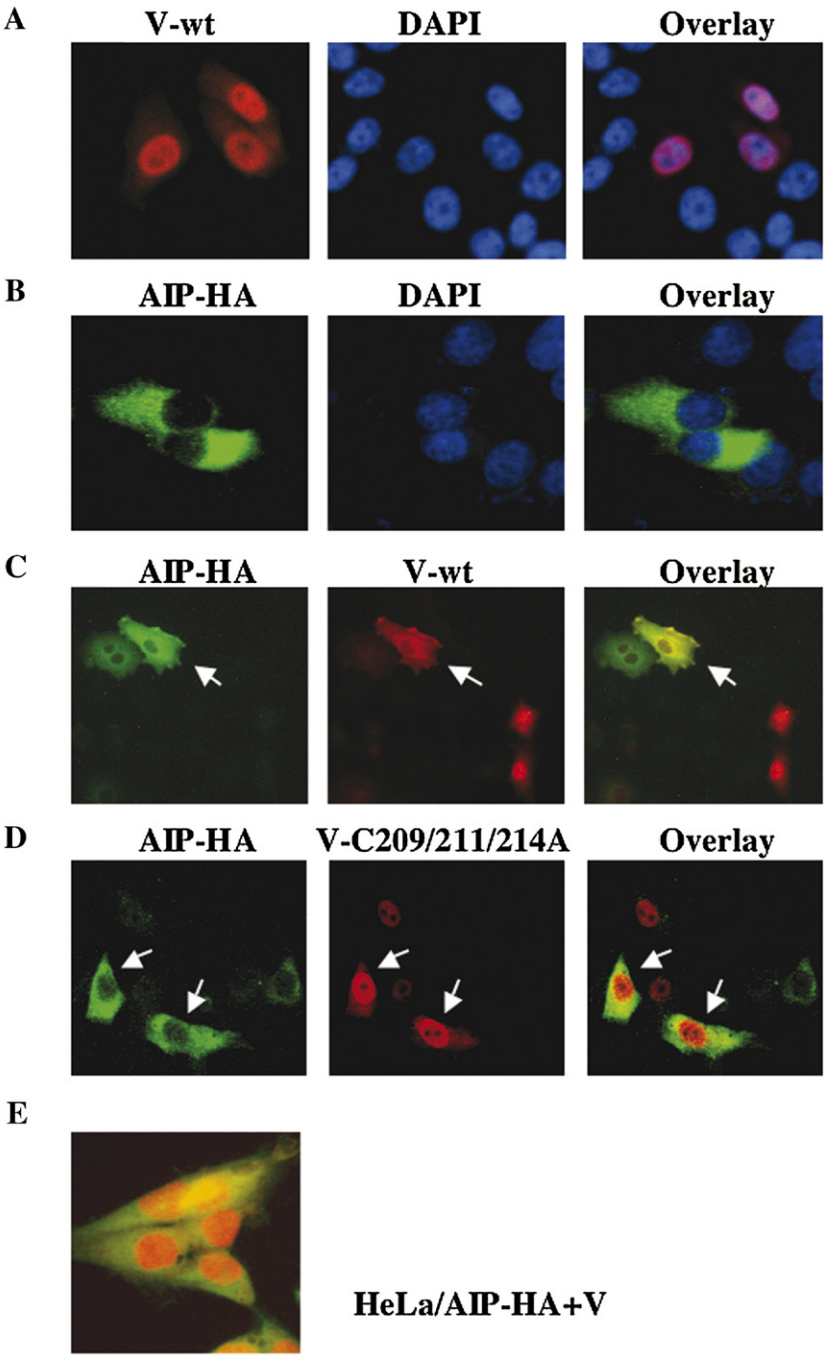

Fig. 7. Immunofluorescence staining patterns of AIP1-HA and V proteins in transiently or constitutively expressing cells. (A) Subcellular distribution of transiently expressed wt V protein. HeLa cells were transfected with plasmid encoding wt V protein. Twenty hours posttransfection, the cells were fixed, permeabilized, and stained with mAb against the V protein. Expressed protein was visualized using TRITC-labeled secondary antibody (red). Nuclei were stained with DAPI (blue). (B) Subcellular distribution of transiently expressed AIP1-HA protein in HeLa cells. Twenty hours posttransfection, the cells were fixed, permeabilized, and stained with mAb against the HA epitope. Expressed protein was visualized using FITC-labeled secondary antibody (green). Nuclei were stained with DAPI (blue). (C and D) Subcellular distribution of transiently expressed AIP1-HA and wt V (C) or mutant V (D) proteins in HeLa cells. Twenty hours posttransfection, the cells were fixed, permeabilized, and stained with anti-P/V (IgG1) and anti-HA (IgG2a) mAbs. Expressed proteins were visualized using TRITC-labeled IgG1 (red) and FITC-labeled IgG2a (green) secondary antibodies. The arrows show the cells expressing both AIP1-HA and V proteins. (E) Subcellular distribution of constitutively expressed AIP1-HA and wt V proteins in HeLa/AIP1-HA+V cells. The cells were fixed, permeabilized, and stained with anti-P/V (IgG1) and anti-HA (IgG2a) mAbs. Expressed proteins were visualized using TRITC-labeled IgG1 (red) and FITC-labeled IgG2a (green) secondary antibodies.

using the same method, we established another cell line, HeLa-tet/shGFP, in which was depleted the GFP protein by adding Dox, as a negative control. The parent hPIV2 growth in HeLa-tet/shGFP was unaffected, whereas the growth in HeLa-tet/shAIP1-1 and 1-2 was inhibited by 10- to 100-fold (Fig. 8B). In contrast, the growth rates of mutant rPIV2s in HeLa-tet/shAIP1-1 treated with/without Dox were similar (Fig. 8C). These results have shown that the association between C-

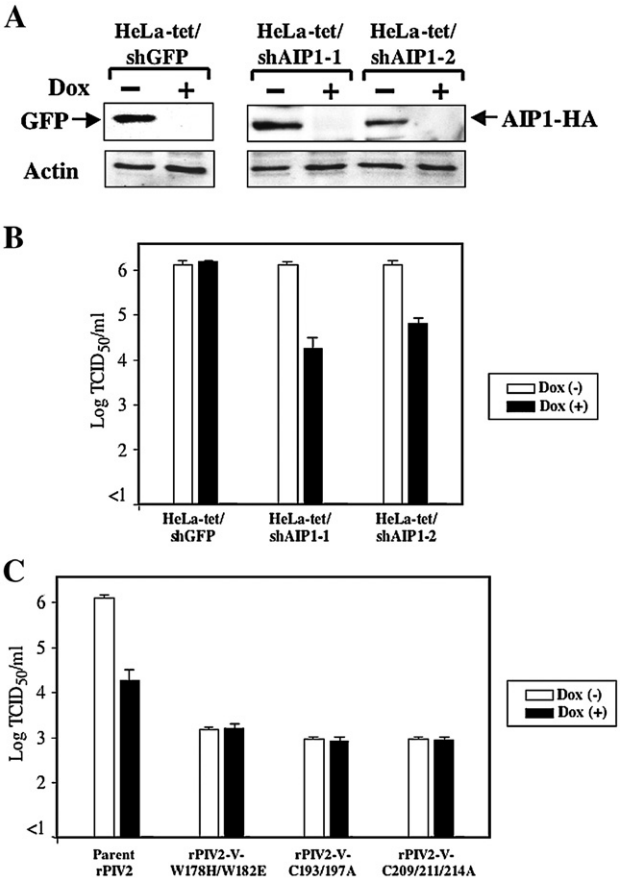

Fig. 8. Virus growth by AIP1 depletion in HeLa-tet/shAIP cells. (A) HeLa-tet/shGFP or HeLa-tet/shAIP1-1 and 1-2 cell lines were transfected with an expression plasmid encoding GFP (pCI-GFP) or AIP1-HA (pCI-AIP-HA), respectively, with/without 5 μg/ml Dox. After 24 h, the cells were assayed by Western blotting using anti-GFP or HA antibody. Actin was used as a loading control. (B) Virus growth of parent rPIV2 in HeLa-tet/shGFP, HeLa-tet/shAIP1-1, and 1-2 cells treated with/without 5 μg/ml Dox at 48 h postinfection. Virus titers were determined as described in the legend for Fig. 1B. (C) Virus growth of parent rPIV2, rPIV2-V-W178H/W182E, -C193/197A, or -C209/211/214A in HeLa-tet/shAIP1-1 cells treated with/without 5 μg/ml Dox at 48 h postinfection. Virus titers were determined as described in the legend for Fig. 1B.

terminus of V protein and AIP1 is important for the virus growth.

Discussion

Our previous study showed that conserved seven Cys residues (193, 197, 209, 211, 214, 218, and 221 Cys), Trp-motif (178, 182, and 192 Trp), 207 Phe of V protein-specific domain, and 143 Phe of the P/V common domain in the hPIV2 V protein are essential for STAT protein degradation and that these residues in the V-specific domain are also essential for promoting virus growth (Nishio et al., 2005b). Recombinant morbilliviruses (Schneider et al., 1997), respiroviruses (Delenda et al., 1997; Kato et al., 1997), and NDV (Huang et al., 2003; Park et al., 2003) that cannot express their V and W proteins have been recovered and all these viruses grow similarly to the respective parent virus at least in some cell lines. In the genus *Rubulavirus*, recombinant SV5 lacking the C-terminus of the V protein (rSV5ΔC) grows well similar to parent rSV5 in Vero cells (He et al., 2002). However, the recombinant PIV2s that have these mutations, C-terminally or fully lacking of V protein, grow poorly even in the IFN-nonproducing or -unresponsive cells, such as Vero, U3A, or U6A. Thus, the V protein of hPIV2 is so clearly important for promoting virus growth.

In this report, we first investigated whether the growth rate of rPIV2s mutant viruses improves in previously established HeLa-V cells that are constitutively expressing the hPIV2 V protein (Nishio et al., 2001). HeLa-V cells contained specifically reduced levels of STAT2, and in these cells, IFN signaling was blocked. Unexpectedly, the growth of rPIV2s mutant viruses could not improve in HeLa-V cells. Huang et al. (2003) reported that transient expression of the carboxyl terminal portion of the V protein enhanced the growth of the NDV mutant viruses in which the expression of the V or both the V and W proteins has been abolished. Thus, we next established a stable HeLa tet-on cell line in which expression of hPIV2 V protein was induced by treatment with Dox (HeLa-tet/V). The virus growth of mutant rPIV2s in HeLa-tet/V cells treated with Dox improved 10- to 100-fold as many as those in the nontreated cells. Although the growth of NDV mutant viruses was similar to that of parent NDV at 24 h after transfection of the V protein (Huang et al., 2003), the yields of rPIV2s mutant viruses were still 10- to 20-fold lower than those of parent rPIV2 and treatment with Dox at 14 h after infection was more effective than treatment with Dox at the same time with infection. Thus, to achieve the similar growth level to parent hPIV2, additional studies including the transient expression at the appropriate time are necessary. In addition, we investigated whether the growth of rPIV2s mutant viruses improves by transiently expressing other rubulavirus V proteins. hPIV2 V protein shows 69.3, 41.1, and 34.8% homologies with those of SV41, SV5, and MuV, respectively (Kawano et al., 1993; Kondo et al., 1990). In these rubulaviruses, only the SV41 V protein has an ability to promote the growth of rPIV2s mutant viruses. Though STAT degradation tropism of these V proteins is different from each other, both V proteins have a common function to promote virus growth.

In previous studies, we identified essential domains for interaction between the hPIV2 NP, P, and V proteins (Nishio et al., 1996, 1997, 1999b; Watanabe et al., 1996b). In these studies, we demonstrated that aa 1–46 of the P/V common region on the V protein are required for its binding to the aa 401–493 on the NP protein, and the V protein may form a complex only with NP^0. In the case of SV5, it has been suggested that the V protein may complete with the P protein for soluble NP, delaying encapsidation and thus being part of a controlling mechanism which switches from virus transcription to replication (Precious et al., 1995). Since the NP and P proteins of rPIV2s that have mutations in the V-specific region show some masses-shaped subcellular distribution in the virus-infected cells, it is predicted that the V-specific region is important in keeping NP soluble for virus growth. In this study, we identified that aa 178–225 on the V-specific region are important to bind to the aa 1–82 on the NP and aa 198–225 on the V-specific domain are important for V–V oligomerization,

but the V protein does not bind directly to the P protein. Therefore, it is supposed that the alteration of the P protein distribution in the mutant rPIV2-infected cells is due to the alteration of the distribution of the NP associated with the P protein. Although V protein oligomerization has been hypothesized to be involved in STAT degradation (Ulane et al., 2005), biological roles of the oligomerization needed to be established. The domains on the NP and V proteins identified in this study and our previous studies are summarized in Fig. 9.

Li et al. (2006) recently reported the crystal structure of complex of DDB1 and SV5 V proteins. The unique C-terminal sequence of the SV5 V protein forms the middle two β strands (β6 and β7) as well as the zinc-binding motif of the core domain, all of which are crucial structure elements for the V protein to maintain its unique fold. It binds two zinc ions via 171 His and seven Cys (190, 194, 206, 208, 211, and 218) residues, all of which are conserved among the V proteins of paramyxoviruses (Paterson et al., 1995). 171 His and three Cys (190, 211, and 218) residues fold one zinc ion, and other four Cys (194, 206, 208, and 211) residues fold another one. From sequence alignment and structural elements of the V proteins of SV5 and hPIV2, two Trp residues (178 and 182) of hPIV2 V protein turned out to locate on the β6 sheet, and 192 Trp on the β7. Therefore, it is speculated that the mutation of Trp-motif on the V protein alters the conformation of these β sheets, and the mutations of Cys-motif alter the novel zinc-finger fold. In this paper, we tested various mutant V proteins, W178H/W182E, C193/197A, C209/211/214A, and C218/221A, to identify the essential amino acids on the V protein for binding to the NP and its oligomerization. As the V protein that has the W178H/W182E mutation could not bind to the NP and V proteins, the β sheets on the V-unique region may be very important for its structure. Since the V83-225/Δ155–192 (this mutant lacks the β sheets and 174 His of the zinc-finger fold on the V-unique region) and the mutant C193/197A (ones of four residues on each zinc-binding sequence are mutated) still have ability to bind with the NP, the structural alteration by mutating each one on zinc-binding residues may be not drastic. However, both of those β sheets and zinc-finger fold are crucial structure elements for the V protein, and both of them would be important for its function.

AIP1 (ALG-2 interacting protein 1)/Alix (ALG-2-interacting protein X) was previously identified as an interactor for the apoptosis-linked-gene 2 (ALG-2) protein and as a calcium binding protein implicated in apoptosis (Missotten et al., 1999; Vito et al., 1999). AIP1 has also been shown to interact with a conserved C-terminal region of human immunodeficiency virus type 1 (HIV-1) p6 and equine infectious anemia virus (EIAV) p9 (Strack et al., 2003). HIV release requires TSG101, a cellular factor that sorts proteins into vesicles that bud into multivesicular bodies (MVB). The proteins involved in MVB biogenesis (the class E proteins) were connected into a coherent network by many different protein–protein interactions, with AIP1 playing a key role in linking complexes that act early (TSG101/ESCRT-1) and late (CHMP4/ESCRT-III) in this pathway. AIP1 interacts specifically with the PT/SAP and LXXLF motifs of HIV-1, and with the YPXL/I-type L domain of EIAV. The V-specific region does not contain any of these motifs required for interaction with AIP1.

Various Gag proteins of retroviruses directly interact to the ubiquitin ligases of the Nedd4 family, which are involved in the ubiquitination of membrane-associated proteins to initiate their sorting toward internal MVB vesicles (Segura-Morales et al., 2005). Since inhibition of virus budding by the dominant-negative form of Vsp4A, an AAA-type ATPase essential for ESCRT function, has been described previously for SV5 and SeV (Sakaguchi et al., 2005; Schmitt et al., 2005), ESCRT may be necessary for efficient budding of paramyxovirus. It has been reported that efficient budding of SV5 virus-like particles (VLPs) requires expression of the NP, and binding between the M protein and AIP1 is not detected (Schmitt et al., 2002, 2005). Since the M protein of hPIV2 binds to the NP (data not shown), the formation of complex between NP, M, and AIP1 may be important for formation of hPIV2 virus particles. Previously, it was shown that the V proteins of SV5 and hPIV2 mediate the STAT protein degradation and have an E3 ubiquitin ligase activity (Ulane and Horvath, 2002). E3s (ubiquitin ligases) are classified into HECT type E3 and RING finger type E3. Nedd4

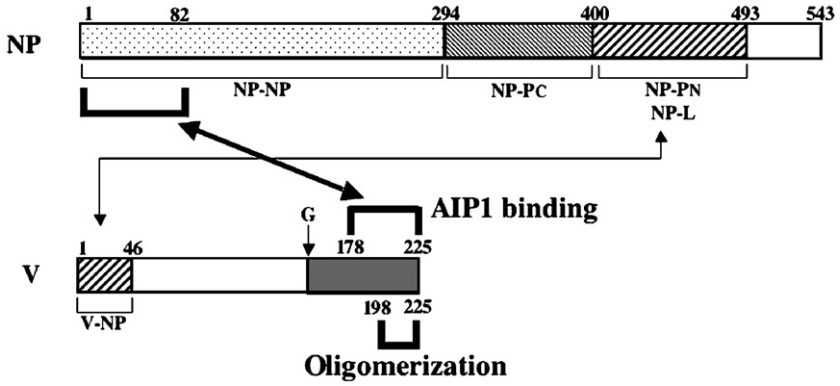

Fig. 9. Schematic model of functional domains in the NP and V proteins of hPIV2. The functional domains identified from this and previous works are indicated by boxes. Amino acid residues 1–46 on the P/V common region are required for binding to the aa 401–493 region on the NP protein. Residues 178–225 on the V-specific domain are important for binding to the aa 1–82 region on the NP protein and AIP1. C-terminal 28 aa on the V-specific domain is required for self-assembly. Residues 1–294, 295–400, and 401–493 on the NP protein are required for self-assembly, for binding to C-terminal domain of P protein, and for binding to the L protein, respectively. The arrow below the letter "G" indicates the editing site.

is HECT type and V protein has RING finger-like motif, which is critical for STAT protein degradation. Thus, it is estimated that the V protein may act as ubiquitin ligase for MBV biogenesis.

The hPIV2 V protein is capable of many protein interactions both with viral partners, including the NP and viral RNA (Nishio et al., 2006), and with cellular machinery, like Mda-5 (Andrejeva et al., 2004) or V-dependent degradation complex components. The conserved carboxyl terminal region (aa 178–225) of the V protein plays an important role in virus growth. The mechanism underlying this phenomenon is not clear yet, but it seems very likely that interactions of the V protein with NP and/or with AIP1 protein through this region are required for efficient virus growth. Since this region harbors the V protein oligomerization domain, the interaction between V proteins might also be involved. In the cells expressing hPIV2 V protein constitutively, the V protein localized in the nuclei and did not show colocalization with AIP1 in the cytoplasm (Fig. 7E), while these proteins colocalized in the cytoplasm when transiently transfected (Fig. 7C). It may account for the observation that the growth of mutant hPIV2s was not improved in HeLa cells constitutively expressing the V protein. AIP1 depletion by shRNA resulted in suppression of growth of hPIV2. Furthermore, overexpression of AIP1 enhanced the growth of mutant hPIV2s (data not shown). Lower growth of mutant rPIV2s may be due to the inability of its mutated V protein to interact with AIP1. We also investigated the association between GST-AIP1 and HA-tagged SV41, SV5, or MuV V proteins by the same method of Fig. 6B. The SV41 and MuV V proteins were associated with GST-AIP1, while SV5 V protein was not (data not shown).

SeV lacking C protein has a severe defect in virus growth (Koyama et al., 2003; Kurotani et al., 1998). The V protein of SeV inhibits RNA replication, whereas the V protein of SV5 inhibits both RNA replication and transcription (Lin et al., 2005). The V protein of SV5 plays an important role in blocking apoptosis (Sun et al., 2004). However, the V proteins of hPIV2, SV5, and SeV and the C protein of SeV are not necessary for recovery of recombinant virus from transfected cDNA clones, though recovery rate of rPIV2 without V protein was very low. At present, it is not clear yet whether the complex of the V, NP, and AIP1 proteins is related to virus budding. Additional studies are required to fully assess the role of the V protein.

Materials and methods

Cells and viruses

HeLa, Vero, COS, human 2fTGH cells, and 2fGH-derived cell lines U3A (STAT1 deficient) and U6A (STAT2 deficient) (gift from Dr. I. M. Kerr, Imperial Cancer Research Fund, London, United Kingdom) were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS). HeLa-V cells, HeLa cell line constitutively expressing the hPIV2 V protein (Nishio et al., 2001), were cultured in Eagle's MEM supplemented with 10% FCS and 1 mg/ml G418 (Geneticin; GIBCO). T-Rex-HeLa cells expressing the Tet-operator were purchased from Invitrogen and cultured according to the manufacturer's instructions. Recombinant human parainfluenza type virus 2 (rPIV2) and various mutant rPIV2, rPIV2-V-W178H/W182E, rPIV2-V-C193/197A, rPIV2-V-C209/211/214A, and rPIV2-V-C218A (Nishio et al., 2005b), were used in this study.

Antibodies

Anti-Flag monoclonal antibody (mAb) was purchased from Sigma (St. Louis, MO). Antibody to GFP (sc-8334) and mAb to actin (sc-8432) and to HA (sc-7392, IgG2a) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA.). mAbs against hPIV2 NP protein (366-1 (reacts with N-terminal 100 aa), 20A (reacts with 308–399 aa), and 159-1 (reacts with C-terminal 50 aa)), against P/V protein (85A, IgG1), and against V protein (53-1V) were previously reported (Nishio et al., 1997, 1999a, 1999b).

Construction of expression plasmids

HPIV2 V gene and hPIV2, SV41, SV5, and MuV V genes fused to N-terminal hemagglutinin (HA) epitope tag were cloned into pcDNA5/TO vector (Invitrogen). Various calmodulin-binding peptide (CBP) fusion protein expression plasmids have been described previously (Nishio et al., 2006). A cDNA fragment encoding the hPIV2 NP, P, V deleted NP, or V mutant was inserted into the plasmid expression vector pcDL-SRα296, as described previously (Nishio et al., 1996, 1997, 2006; Takebe et al., 1988). Flag-tagged hPIV2 V protein expression plasmid has been described previously (Nishio et al., 2005a). The full-length cDNA clone of AIP1/Alix (GeneBank accession numbers NM 013374) was subcloned into pCI-neo vector (Promega) with a HA tag at the C-terminus (pCI-AIP-HA). For glutathione *S*-transferase (GST)-AIP1 fusion protein, the cDNA of the AIP1 protein was subcloned into pGEX-5X (Pharmacia). All constructs were confirmed by nucleic acid sequencing.

Establishment of the Doxycycline (Dox)-inducible hPIV2 V or HA-tagged V HeLa cell lines

To obtain the Dox (Clontech)-inducible V cell lines, T-Rex-HeLa cells were transfected with the pcDNA5/TO-based constructs by using FuGENE 6 (Roche) according to the manufacturer's instructions. At 2 days after transfection, the culture media were changed to MEM containing 10% FCS, 200 μg/ml hygromycin (Invitrogen), 5 μg/ml blasticidin (Funakoshi, Japan), and 0.2% agarose, and the cells were cultured for 3 weeks. Five independent clones that stably exhibited high expression levels were analyzed. The Tet-on hPIV2 V cell line (HeLa-tet/V), the Tet-on HA-tagged hPIV2 V cell line (HeLa-tet/HA-PIV2V), the Tet-on HA-tagged SV5 V cell line (HeLa-tet/HA-SV5V), the Tet-on HA-tagged SV41 V cell line (HeLa-tet/HA-SV41V), and the Tet-on HA-tagged mumps virus V cell line (HeLa-tet/HA-MuV) were maintained in Eagle's MEM supplemented with 10% FCS, 100 μg/ml hygromycin, and 5 μg/ml blasticidin.

Establishment of cell lines constitutively expressing both hPIV2 V and AIP1-HA proteins

To obtain cell lines expressing both hPIV2 V and AIP1-HA proteins, HeLa cells were transfected with the plasmids, pDS-V and pCI-AIP-HA, by using FuGENE 6 according to the manufacturer's instructions. At 2 days after transfection, the culture media were changed to MEM containing 10% FCS, 1 mg/ml G418, and 0.2% agarose, and the cells were cultured for 3 weeks. Five independent clones that exhibited high expression levels were analyzed. The HeLa/AIP-HA+V cell line was maintained in Eagle's MEM supplemented with 10% FCS and 1 mg/ml G418.

Viral growth kinetics

Various monolayer cells were infected with viruses at a multiplicity of infection (MOI) of about 0.1 and incubated at 37 °C in MEM with 2% FCS. Supernatants were harvested at appropriate intervals, and virus titers were determined by CPE method using Vero cells and expressed as 50% tissue culture infectious dose ($TCID_{50}$) at 3 days postinfection.

Immunofluorescence staining

The cells were fixed with 3% paraformaldehyde for 30 min at room temperature and rinsed twice with phosphate-buffered saline (PBS). The cells were permeabilized with PBS–0.05% Tween-20 (PBS-T) for 30 min and washed twice with PBS. The cells were then incubated for 60 min with primary antibody and washed three times with PBS. Next, the cells were incubated for 60 min with the appropriate secondary antibodies [TRITC-labeled goat anti-mouse IgG1 and FITC-labeled rat anti-mouse IgG2a (Southern Biotechnology Associates Inc.)] and washed with PBS. Immunofluorescently stained cells were analyzed using a fluorescence microscope.

Purification of bacterially expressed proteins

Bacterially expressed CBP fusion proteins were purified by the method similar to that described previously (Nishio et al., 1999a). AIP1 protein was expressed in *Escherichia coli* BL21 cells as GST fusion protein under isopropyl-1-thio-β-D-galactopyranoside induction. The GST and GST-AIP1 fusion proteins were purified from bacteria by using glutathione–Sepharose beads (Amersham Biosciences, Inc.) according to the manufacturer's instructions.

Cell extraction, immunoblotting, immunoprecipitation, and GST-pulldown assay

For preparation of cell extracts, cells were lysed in lysis buffer (50 mM Tris–HCl (pH 7.5), 40 mM NaCl, 0.6% NP40, and 4 mM phenylmethylsulfonyl fluoride). For immunoblotting, cell extracts were separated by SDS–PAGE, transferred to a nitrocellulose membrane, and analyzed by a Western blot technique with appropriate antibodies as described previously (Nishio et al., 2002). For immunoprecipitation of Flag epitope-tagged proteins, cell extracts were incubated with an anti-Flag M2 agarose affinity gel (Sigma, St. Louis, MO) for 6 h at room temperature. The agarose beads were washed three times with lysis buffer and then extracted with SDS–PAGE sample buffer for analysis by a Western blotting technique with appropriate antibodies. For GST-pulldown assay, whole-cell extracts were incubated with purified GST or GST-AIP1 fusion protein–agarose beads in IP buffer (50 mM Tris–HCl (pH 7.5), 5 mM EDTA, 1% Triton X-100, and 4 mM phenylmethylsulfonyl fluoride) for 8 h and then washed five times with IP buffer. Bound proteins were eluted by boiling in protein gel loading buffer and separated by SDS–PAGE for Western blotting.

Depletion of AIP1/Alix by expressing short hairpin RNA (shRNA) in Dox-inducible manner

Two DNA fragments encoding shRNA against AIP1/Alix were generated by PCR and subcloned into the pH1dTO vector (Takei et al., 2006). pH1dTO-shAIP1-1 and pH1dTO-shAIP1-2, which target nucleotides 1988-GAATTACTGCAACGAAAT-2005 and 2429-GCTCAAGATGGTGTGATAAAT-2449, respectively, were constructed, and pH1dTO-shGFP, which targets to the green fluorescent protein (GFP) transcript, was constructed as a negative control. To obtain the Dox-inducible shRNA cell lines, T-Rex-HeLa cells were transfected with the pH1dTO-shAIP1-1, pH1dTO-shAIP1-2, or pH1dTO-shGFP with a Linear Hygromysin Maker™ (Clontech) by using FuGENE 6 as described above. To assess the depletion of AIP1/Alix or GFP, HeLa-tet/shAIP1-1, HeLa-tet/shAIP1-2, or HeLa-tet/shGFP cells were transfected with the expression plasmid for AIP1-HA (pCI-AIP-HA) or GFP (pCI-GFP) with/without 5 μg/ml Dox. Two independent clones for each construct that exhibited most efficient knockdown were analyzed.

Acknowledgments

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